CHOLINE MONOOXYGENASE GENE

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to plant choline monooxygenase which is involved in the occurrence of plant injury attributable to dry or saline soil, which is believed to contribute to the improvement of plant tolerance to dry or saline soil, and which is induced by dry or saline soil, as well as a gene encoding the choline monooxygenase.

2. Description of the Related Art

At present, desertification or salt accumulation in cultivated land is progressing in a large number of areas on the earth. These environmental changes are considered as serious issues in connection with current environmental problems and food problems in the 21st century. As means to solve these problems, the breeding of environmental stress-resistant plants is attracting attention together with engineering-based solutions such as irrigation.

Specific examples of damage caused by salt accumulation may be enumerated as follows: (1) because of accumulated salts in soil, moisture potential in soil decreases, which makes it impossible for plants to absorb moisture; (2) because of salts which have been absorbed (or which have invaded) into plant bodies, plant metabolism is disturbed; and (3) because of accumulated salts, absorption of other ions necessary for plant growth is inhibited (Fumihiko Sato, Plant Cell Engineering, an extra issue, "Environmental Problems and Biotechnology", pp. 33-39, 1994). In particular, inhibition of moisture absorption caused by dehydration, salt injury, etc. eventually decreases photosynthesis activity to thereby inhibit the growth of plants greatly.

The existence of adaptation mechanisms against stresses such as dehydration or salts has become evident in microorganisms and plants. Among all, compatible solutes (i.e. low molecular weight organic compounds or osmoregulating substances) have been investigated

vigorously. Compatible solutes are those substances which are characterized by having low molecular weights, being rich in water-solubility and difficult to metabolize, and not affecting metabolism. As specific examples of compatible solutes, amphoteric compounds such as glycine betaine, proline, and polyols such as pinitol, sorbitol, mannitol are known. In particular, glycine betaine (hereinafter, referred to as "betaine") is utilized widely not only in higher plants such as chenopodiaceous plants, gramineous plants and solanaceous plants, but also in microorganisms. It is reported that this compatible solute is functioning in protecting proteins from high temperature stress (Paleg, L.G. et al., Aust. J. Plant Physiol. 8:107-114, 1981; Allakhverdiev S.I., J. Photochem. Photoiol. 34:149157, 1996), in maintaining osmotic pressure balance against the environment (Robinson, S.P. and Jones, G.P., Aust. J. Plant Physiol. 13:659-668, 1986) and in protecting soluble enzymes from salt stress (Gabbay-Azaria et al., Arch. Biochem. Biophys. 264:333-339, 1988).

In spinach which is well studied among higher plants, betaine is synthesized in two steps through choline and betaine aldehyde. Specifically, oxidization in the first step is catalyzed by a ferredoxin-dependent choline monooxygenase (Brouquisse, R. et al., Plant Physiol. 90: 322-329, 1989), and oxidization in the second step is catalyzed by a NAD-dependent betaine aldehyde dehydrogenase (Weretilnyk, E.A. et al., Planta. 178: 342-352, 1989). It is confirmed that when such a plant is exposed to salt stress, the activity of each of the above enzymes rises and the amount of betaine is increased (Hanson, A.D. et al., Proc. Natl. Acad. Sci, U.S.A. 82: 3678-3682, 1985).

A choline oxidase obtained from a Gram-negative soil bacterium *Arthrobacter globiformis* is able to oxidize choline to betaine in one step oxidization (Ikuta, S. et al., J. Biochem. 82: 1741-1749, 1977).

Several attempts have been made to accumulate betaine in plants and confer salt tolerance on them by incorporating in plant bodies two enzyme genes from *Escherichia coli* and a higher plant or a choline oxidase gene and allowing the gene constant expression. Accumulation of betaine in plant bodies have been reported when *Arthrobacter globiformis* codA gene

(WO96/29857), *E. coli* betA gene (Japanese Unexamined Patent Publication No. 10-191983) and spinach CMO gene (Nuccio, M.L. et al., The Plant J., 16: 487-496, 1998) were incorporated. However, no attempts have succeeded in accumulating betaine at such levels as seen in salt tolerant plants. Thus, it is desired to establish a betaine synthesis system which can accumulate betaine in plants at high levels.

OBJECTS AND SUMMARY OF THE INVENTION

It is the object of the invention to provide a choline monooxygenase, a gene encoding the same, a vector comprising the gene, a transformant comprising the vector, a stress resistant plant, and a method for inducing betaine accumulation.

As a result of intensive and extensive researches toward the solution of the above problem, the present inventors have isolated a full-length choline monooxygenase gene inducible by dry and saline soil from *Chenopodium album* L. which exhibits tolerance to dry and saline soil and can accumulate betaine at a high rate of about 60 μ mol/g fresh weight under salt stress, and found that this gene is capable of accumulating betaine in plant bodies. Also, the inventors have found for the first time that a transit peptide sequence of the choline monooxygenase gene induces protein accumulation under salt stress. Thus, the present invention has been achieved.

The present invention relates to the following recombinant protein (a) or (b):

(a) a protein comprising the amino acid sequence shown in SEQ ID NO: 2, 4 or 6;

(b) a protein which comprises the amino acid sequence shown in SEQ ID NO: 2, 4 or 6 having deletion, substitution or addition of one or several amino acids, and which has choline monooxygenase activity.

The present invention further relates to a choline monooxygenase gene encoding the above-described protein.

The present invention further relates to a gene comprising the following DNA (c) or (d):

(c) a DNA comprising the nucleotide sequence shown in SEQ ID NO: 1, 3 or 5;

(d) a DNA which hybridizes to a DNA comprising the nucleotide sequence shown in SEQ ID NO: 1, 3 or 5 under stringent conditions and which encodes a protein having choline monooxygenase activity.

The present invention further relates to a recombinant vector comprising the above-described gene.

The present invention further relates to a transformant comprising the above-described recombinant vector.

The present invention further relates to a method for producing a choline monooxygenase, comprising culturing the above-described transformant and recovering the choline monooxygenase from the resultant culture.

The present invention further relates to the following peptide (e) or (f):

(e) a peptide comprising the amino acid sequence shown in SEQ ID NO: 17;

(f) a peptide which comprises the amino acid sequence shown in SEQ ID NO: 17 having deletion, substitution or addition of one or several amino acids and which has signal peptide activity; or a salt thereof.

The present invention further relates to a gene encoding the above-described peptide. Specific examples of the gene include a gene comprising the following DNA (g) or (h):

(g) a DNA comprising the nucleotide sequence shown in SEQ ID NO: 16;

(h) a DNA which hybridizes to a DNA comprising the nucleotide sequence shown in SEQ ID NO: 16 under stringent conditions and which encodes a protein having signal peptide activity.

The present invention further relates to a recombinant vector comprising a gene encoding the above-described peptide and a gene of interest. As the gene of interest, a gene which leads to production of a polypeptide or production of a plant metabolite (e.g. a substance that confers stress resistance), or *Chenopodium album* choline monooxygenase gene may be enumerated.

The present invention further relates to a transformant comprising the recombinant vector comprising a gene encoding the above-described peptide and a gene of interest. Specific

examples of the transformant include a plant body, plant organ, plant tissue and cultured plant cell.

The present invention further relates to a method for creating an environmental stress-resistant plant, comprising culturing or cultivating a transformed plant comprising the above-described recombinant vector under environmental stress (e.g. salt stress) conditions; or an environmental stress-resistant plant created by this method.

The present invention further relates to a method for inducing accumulation of a polypeptide or a plant metabolite (e.g. a substance that confers environmental stress resistance), comprising culturing or cultivating the above-described transformant under environmental stress conditions. As a specific example of the substance that confers environmental stress resistance, betaine may be given.

The present invention further relates to a method for producing betaine, comprising culturing or cultivating a transformant comprising the recombinant vector comprising a gene encoding the above-described peptide and a choline monooxygenase gene and then recovering betaine from the resultant culture or the cultivated product.

This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No.11-273275, which is a priority document of the present application.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram showing the responsivity of a choline monooxygenase (CMO) transit peptide to salt stress.

Fig. 2 is a graph showing betaine accumulation in a transgenic tobacco.

DETAILED DESCRIPTION OF THE INVENTION

Hereinbelow, the present invention will be described in detail.

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The present invention relates to plant choline monooxygenase gene which is induced by dry and saline soil. As one example of such a gene, *Chenopodium album* choline monooxygenase gene will be described. However, it is believed that other choline monooxygenase genes inducible by dry and saline soil exist in other plant species which, like *Chenopodium album*, exhibit tolerance to dry and saline soil. Thus, choline monooxygenase genes derived from plants other than *Chenopodium album* are also included in the gene of the invention.

The choline monooxygenase of the invention comprises the amino acid sequence shown in SEQ ID NO: 2, 4 or 6. However, these amino acid sequences may have some difference among plant varieties. Also, even in the same plant variety, the amino acid sequence of choline monooxygenase may be varied because of mutations or the like. Accordingly, a protein which comprises the amino acid sequence shown in SEQ ID NO: 2, 4 or 6 having deletion, substitution or addition of one or several (e.g. one to ten) amino acids, and which has choline monooxygenase activity is also included in the present invention.

Further, the present invention provides a choline monooxygenase gene comprising the nucleotide sequence shown in SEQ ID NO: 1, 3 or 5. However, the gene of the invention is not limited to these genes but includes all of the genes encoding the amino acid sequence shown in SEQ ID NO: 2, 4 or 6. The gene of the invention also includes all of the genes encoding a substantial choline monooxygenase comprising the amino acid sequence shown in SEQ ID NO: 2, 4 or 6 having substitution, deletion or addition of one or several (e.g. one to ten) amino acids.

1. Cloning of the Gene of the Invention

The gene of the invention can be isolated by extracting RNA from a plant loaded with stress (e.g. dehydration or salt treatment) and then subjecting the resultant RNA to RT-PCR. Specific examples of plants which may be used as a source of mRNA include, but are not limited to, chenopodiaceous plants to which *Chenopodium album* belongs. The preparation of mRNA may be performed by conventional methods. For example, total RNA may be extracted from the above-mentioned source by the guanidium thiocyanate-cesium chloride

method or the like, and then poly(A)+ RNA (mRNA) may be obtained therefrom by affinity column method using oligo dT-cellulose or poly U-Sepharose or batch method. The poly(A)+ RNA may be fractionated further by sucrose gradient centrifugation or the like. Using the thus obtained mRNA as a template, single-stranded cDNA is synthesized with oligo dT primers and a reverse transcriptase. Then, double-stranded cDNA is synthesized from the single-stranded cDNA. As a pair of primers to be used in RT-PCR, oligonucleotides corresponding to two portions of other plant's choline monooxygenase which are highly homologous among plant choline monooxygenases may be used (e.g. partial sequences from spinach choline monooxygenase).

Further, a cDNA fragment encoding a part of the choline monooxygenase of interest is cloned from the cDNA by RT-PCR. From this cDNA fragment, primers for RACE-PCR are prepared. Using these primers, RACE-PCR is performed on a template cDNA to which an adaptor is ligated at both ends (RACE). Thus, a cDNA encoding the full-length of the choline monooxygenase of interest can be obtained. RACE (Rapid Amplification of cDNA Ends) is a method for recovering the 5' or 3' missing portion of a cDNA rapidly.

More specifically, upon determination of the sequence of the partial cDNA fragment obtained by RT-PCR, gene specific primers (GSPs) are designed based on the resultant partial cDNA sequence. Gene specific primers are primers necessary for amplifying DNA fragments which are located at 5' and 3' flanking regions of the above-mentioned partial cDNA sequence and whose sequences are unknown. GSP sequences may be selected arbitrarily from the above-mentioned partial cDNA sequence.

Subsequently, DNA fragments located on the 5' side (upstream) and the 3' side (downstream) of the above-mentioned partial cDNA are amplified. Although the sequences of these DNA fragments which serve as templates are unknown, an adaptor sequence is ligated to one end of each fragment. Then, using a primer which hybridizes to the adaptor (termed "adaptor primer (AP)") and the above-described GSP as a pair of primers, the adaptor-ligated cDNA fragment whose sequence is unknown is amplified.

In the present invention, RACE may be performed using a commercial kit (Marathon™

cDNA Amplification Kit; Clontech).

The nucleotide sequences of the resultant cDNA fragments are determined by a method based on PCR. For example, a reaction is performed using PRISM Sequencing Kit (Perkin Elmer) containing fluorescence dideoxy terminator, followed by determination of the nucleotide sequence of the resultant product with an autosequenser (e.g. Model ABI 373; Applied Biosystems).

From the thus obtained partial sequence and nucleotide sequences of 5' and 3' RACE products, the nucleotide sequence of the full-length cDNA can be obtained by assembling. Briefly, by joining these fragments at the sites overlapping with each other, the full-length nucleotide sequence containing 5' and 3' ends is obtained.

SEQ ID NOS: 1, 3 and 5 illustrate nucleotide sequences of the gene of the invention. SEQ ID NOS: 2, 4 and 6 illustrate amino acid sequences of the choline monooxygenase of the invention. However, as long as a protein comprising one of the above amino acid sequences has choline monooxygenase activity, the amino acid sequence may have variation (such as deletion, substitution or addition) in one or several amino acids.

For example, 1 to 10 amino acids, preferably 1 to 5 amino acids of the amino acid sequence shown in SEQ ID NO: 2, 4 or 6 may be deleted; 1 to 10 amino acids, preferably 1 to 5 amino acids may be added to the amino acid sequence shown in SEQ ID NO: 2, 4 or 6; or 1 to 10 amino acids, preferably 1 to 5 amino acids of the amino acid sequence shown in SEQ ID NO: 2, 4 or 6 may be substituted with other amino acids.

The term "choline monooxygenase activity" used in the present invention means the activity to catalyze the first step oxidization from choline to betaine aldehyde. The presence or absence of the above-described activity of the protein of the invention can be confirmed by adding to a crude extract of a plant a solution containing choline chloride and DCPIP, and measuring changes in absorbance in the resultant reaction solution (Japanese Unexamined Patent Publication No. 10-191983).

Further, a DNA which hybridizes to the above-described gene under stringent conditions is also included in the gene of the invention. The "stringent conditions" means those conditions

under which the so-called specific hybrid is formed but non-specific hybrids are not formed. For example, those conditions under which highly homologous DNAs (i.e. DNAs having 60% homology or more, preferably 80% homology or more) hybridize to each other and DNAs with less homology do not hybridize to each other may be given. More specifically, stringent conditions means a sodium concentration of 150-900 mM, preferably 600-900 mM, and a temperature of 60-68 ℃, preferably 65 ℃.

When genes consisting of the nucleotide sequences shown in SEQ ID NOS: 1, 3 and 5, respectively, are designated type A, type B and type C, there are 97.0% homology between type A and type B, 98.2% homology between type A and type C, and 97.5% homology between type B and type C. Therefore, a gene which comprises a nucleotide sequence having 90%, preferably 97% homology or more to type A gene and which encodes a protein having choline monooxygenase activity is also included in the gene of the invention.

Once the nucleotide sequence of the gene of the invention has been determined, the gene of the invention can be obtained by chemical synthesis, by PCR using the cloned cDNA as a template, or by hybridization using a DNA fragment having the nucleotide sequence as a probe. Further, a modified DNA encoding the choline monooxygenase may be synthesized by site specific mutagenesis or other techniques.

In order to introduce mutations into genes, known techniques such as the method of Kunkel, the gapped duplex method, etc. or techniques based on these methods may be used. For example, mutations may be introduced using a mutation introduction kit (e.g. Mutant-K or Mutant-G both from Takara) utilizing site specific mutagenesis or LA PCR in vitro Mutagenesis series kits (Takara).

2. Preparation of Recombinant Vectors and Transformants

(1) Preparation of Recombinant Vectors

The recombinant vector of the invention can be obtained by ligating (inserting) the gene of the invention to (into) an appropriate vector. The vector into which the gene of the invention is to be inserted is not particularly limited as long as it is replicable in a host. For

example, plasmid DNA, phage DNA or the like may be used.

Specific examples of plasmid DNA include *E. coli*-derived plasmid s (e.g. pBR322, pBR325, pUC118, pUC119, etc.), *Bacillus subtilis*-derived plasmids (e.g. pUB110, pTP5, etc.) and yeast-derived plasmids (e.g. YEp13, YEp24, YCp50, etc.), and specific examples of phage DNA include λ phage (Charon4A, Charon21A, EMBL3, EMBL4, λgt10, λgt11, λZAP, etc.). Further, an animal virus vector such as retrovirus or vaccinia virus; or an insect virus vector such as baculovirus may also be used.

For insertion of the gene of the invention into a vector, a method may be employed in which the purified DNA is digested with appropriate restriction enzymes and then inserted into the restriction site or the multi-cloning site of an appropriate vector DNA for ligation.

The gene of the invention should be incorporated into the vector so that the function thereof can be manifested. For this purpose, the vector of the invention may contain, if desired, cis elements such as an enhancer, splicing signals, poly(A) addition signals, selection markers, ribosome binding sequences (SD sequences) or the like in addition to a promoter and the gene of the invention. As the selection marker, dihydrofolate reductase gene, ampicillin resistance gene, neomycin resistance gene or the like may be enumerated.

(2) Preparation of Transformants

The transformant of the invention can be obtained by introducing the recombinant vector of the invention into a host so that the gene of interest can be expressed. The host useful in the invention is not particularly limited as long as it can express the DNA of the invention. Specific examples of the host include *Escherichia* bacteria such as *Escherichia coli*; *Bacillus* bacteria such as *Bacillus subtilis*; *Pseudomonas* bacteria such as *Pseudomonas putida*; *Rhizobium* bacteria such as *Rhizobium meliloti*; yeast such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*; animal cells such as COS cells, CHO cells; or insect cells such as Sf9 cells.

When a bacterium such as *E. coli* is used as the host, the recombinant vector of the invention should be capable of autonomous replication in this microorganism and, at the same time, it is preferred that the vector be composed of a promoter, a ribosome binding sequence, the

gene of the invention and a transcription termination sequence. The vector may also contain a gene(s) to control the promoter.

As *Escherichia* bacteria, *E. coli* DH5 α or Y1090 strain may be used, for example. As *Bacillus* bacteria, *Bacillus subtilis* may be used, for example. However, the present invention is not limited to these bacteria.

As the promoter, any promoter may be used as long as it can direct the expression of the gene of interest in a host such as *E. coli*. For example, an *E. coli*- or phage-derived promoter such as trp promoter, lac promoter, P_L promoter or P_R promoter may be used. An artificially designed and altered promoter such as tac promoter may also be used.

As a method for introducing the recombinant vector into a bacterium, any method of DNA transfer into bacteria may be used. For example, a method using calcium ions [Cohen, S.N. et al., Proc. Natl. Acad. Sci., USA, 69:2110-2114 (1972)], electroporation or the like may be used.

When yeast is used as the host, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* or the like may be used. In this case, the promoter to be used is not particularly limited. Any promoter may be used as long as it can direct the expression of the gene of interest in yeast. For example, gal1 promoter, gal10 promoter, heat shock protein promoter, MF α 1 promoter, PH05 promoter, PGK promoter, GAP promoter, ADH promoter, AOX1 promoter or the like may be used.

As a method for introducing the recombinant vector into yeast, any method of DNA transfer into yeast may be used. For example, electroporation [Becker, D.M., Methods Enzymol., 194:182-187 (1990)], the spheroplast method [Hinnen, A. et al., Proc. Natl. Acad. Sci., USA, 75:1929-1933 (1978)], the lithium acetate method [Itoh, H., J. Bacteriol., 153:163-168 (1983)] or the like may be employed.

When an animal cell is used as the host, simian COS-7 or Vero cells, Chinese hamster ovary cells (CHO cells), mouse L cells, rat GH3 cells, human FL cells or the like may be used. As a promoter, SR α promoter, SV40 promoter, LTR promoter, CMV promoter or the like may be used. The early gene promoter of human cytomegalovirus may also be used. As a method

for introducing the recombinant vector into the animal cell, electroporation, the calcium phosphate method, lipofection or the like may be employed.

When an insect cell is used as the host, Sf9 cells or the like may be used. As a method for introducing the recombinant vector into the insect cell, the calcium phosphate method, lipofection, electroporation or the like may be employed.

When a plant is used as the host, a transformant may be prepared as described below.

In the present invention, a plant to be transformed may be any of the following plant materials: entire plant bodies, plant organs (e.g. leaves, petals, stems, roots, seeds, etc.), plant tissues (e.g. epidermis, phloem, parenchyma, xylem, vascular bundles, palisade tissues, spongy tissues, etc.) or cultured plant cells. Specific examples of plant species which may be used for transformation include, but are not limited to, those belonging to the genus *Chenopodiaceae*, *Solanaceae*, *Gramineae*, *Leguminosae*, *Rosaceae*, *Compositae*, *Liliaceae*, *Caryophyllaceae*, *Cucurbitaceae*, *Convolvulaceae* or *Cruciferae*.

The above-described recombinant vector may be introduced into a plant by conventional transformation methods, e.g. the *Agrobacterium* method, the particle gun method, PEG method, electroporation, etc. For example, when the *Agrobacterium* method is used, a plant expression vector constructed is transferred into an appropriate *Agrobacterium* strain (e.g. *Agrobacterium tumefaciens* LBA4404), followed by infection of aseptically cultured leaf discs of a host (e.g. tobacco) with this strain according to the leaf disc method (Hirobumi Uchimiya, Operation Manual for Plant Genes, 1990, pp. 27-31, Kohdansha Scientific Co., Ltd., Tokyo). Thus, a transformed tobacco is obtained.

When the particle gun method is used, entire plant bodies, plant organs or plant tissues may be used as they are, or may be used after preparation of pieces or protoplasts. The thus prepared samples may be bombarded using a gene transfer apparatus (e.g. PDS-1000; BioRad). Bombardment conditions vary depending on the type of the plant or sample. Usually, the sample is bombarded under a pressure of about 450-2000 psi and at a distance of 4-12 cm.

When a cultured plant cell is used as the host, transformation is performed by introducing the recombinant vector thereinto by the particle gun method, electroporation or the like.

Tumor tissues, shoots, hairy roots, etc. resulted from the transformation can be used directly in cell culture, tissue culture or organ culture. Further, they can be regenerated to plant bodies by using conventional plant tissue culture methods and administering plant hormones (e.g. auxin, cytokinin, gibberellin, abscicic acid, ethylene, brasinolide) at appropriate concentrations.

Whether the gene of interest has been integrated into the host or not can be confirmed by PCR, Southern hybridization, Northern hybridization or the like. For example, DNA is prepared from the transformant and then DNA specific primers are designed for PCR. A PCR reaction may be performed under the same conditions as described above in the preparation of plasmids. Subsequently, the amplified product is subjected to agarose gel electrophoresis, polyacrylamide gel electrophoresis or capillary electrophoresis and stained with ethidium bromide, SYBR Green solution, etc. By detecting the amplified product as a single band, it can be confirmed that the host has been transformed. Alternatively, a PCR reaction may be performed using primers labelled with a fluorescent dye or the like, and then the amplified product may be detected. Further, a method may be employed in which a PCR amplified product is bound to a solid phase such as a microplate, and then the product is confirmed by fluorescence or enzyme reaction.

3. Production of the Protein of the Invention

The protein of the invention is a protein comprising the amino acid sequence encoded by the choline monooxygenase gene of the invention; or a protein which comprises the above amino acid sequence having the above-described mutation in a plurality of amino acids and yet which has choline monooxygenase activity. In this specification, the protein of the invention is sometimes called the "choline monooxygenase protein".

The choline monooxygenase protein of the invention can be obtained by culturing the above-described transformant in a medium and recovering the protein from the resultant culture. The term "culture" means any of the following materials: culture supernatant, cultured cells or microorganisms, or disrupted cells or microorganisms.

The culturing of the transformant of the invention is carried out by conventional methods used for culturing hosts.

As a medium for culturing the transformant obtained from a microorganism host such as *E. coli* or yeast, either a natural or synthetic medium may be used as long as it contains carbon sources, nitrogen sources and inorganic salts assimilable by the microorganism and is capable of efficient culturing of the transformant.

As carbon sources, carbohydrates such as glucose, fructose, sucrose, starch; organic acids such as acetic acid, propionic acid; and alcohols such as ethanol, propanol may be used.

As nitrogen sources, ammonium salts of inorganic or organic acids (e.g. ammonia, ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate, etc.) and other nitrogen-containing compounds (e.g. Peptone, meat extract, corn steep liquor, etc.) may be used.

As inorganic substances, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, iron(II) sulfate, manganese sulfate, copper sulfate, calcium carbonate and the like may be used.

Usually, the culture is carried out under aerobic conditions used in shaking culture or aeration agitation culture, at 37℃. Adjustment of the pH of the medium is carried out with an inorganic or organic acid, an alkali solution or the like.

During the culture, antibiotics such as ampicillin or tetracycline may be added to the medium if necessary.

When a microorganism transformed with an expression vector containing an inducible promoter is cultured, an inducer may be added to the medium if necessary. For example, when a microorganism transformed with an expression vector containing Lac promoter is cultured, isopropyl-β-D-thiogalactopyranoside (IPTG) or the like may be added. When a microorganism transformed with an expression vector containing trp promoter is cultured, indoleacetic acid (IAA) or the like may be added.

As a medium for culturing the transformant obtained from an animal cell as a host,

commonly used RPMI1640 medium or DMEM medium, or one of these media supplemented with fetal calf serum, etc. may be used. Usually, the culture of such a transformant is carried out in the presence 5% CO_2 at 37℃ for 1 to 30 days. During the culture, antibiotics such as kanamycin or penicillin may be added to the medium if necessary.

After the culture, the choline monooxygenase protein of the invention is extracted by disrupting the cultured microorganisms or cells if the protein is produced in the microorganisms or cells. If the protein of the invention is produced outside of the microorganisms or cells, the culture fluid is used as it is or subjected to centrifugation to remove the microorganisms or cells. Thereafter, the resultant supernatant is subjected to conventional biochemical techniques used for isolating/purifyinng a protein. These techniques include ammonium sulfate precipitation, gel chromatography, ion exchange chromatography and affinity chromatography; these techniques may be used independently or in an appropriate combination to thereby isolate and purify the protein of the invention from the above culture.

When the host is a plant, the choline monooxygenase of the invention can be produced by culturing or cultivating the transformed plant. Further, it is also possible to produce the product of a reaction catalyzed by the choline monooxygenase, or the intermediates and/or the final product (e.g. betaine) of a series of biosynthesis reactions following the above reaction.

When the transformant is a plant cell or plant tissue, culture may be performed in a conventional plant culture medium, e.g. MS basal medium (Murashige, T. & Skoog. F. (1962) Physiol. Plant. 15: 473), LS basal medium (Linsmaier, E.M. & Skoog, F. (1965) Physiol. Plant. 18: 100), or protoplast culture medium (modified LS medium). Conventional solid culture methods may be used, but it is preferable to use liquid culture methods.

A transformed plant cell, tissue or organ is inoculated into the above medium at a rate of 0.1-2.0 g fresh weight/liter. If necessary, NAA, 2.4-D, BA, kinetin or the like is added to the medium appropriately. Then, the transformant is cultured. The pH of the medium at the start of culture is adjusted to 5-7. Usually, the culture is performed at 20-30 ℃, preferably at around 25 ℃, under aeration at 0.2-1 vvm and agitation at 50-200 rpm for 1-6 weeks.

When the transformant is a plant body, it may be cultivated on a field or in a glass house

or may be hydroponically cultured.

In order to recover the protein of the invention from cultured cell or tissues, first, the cells are disrupted by cell lysis treatment using an enzyme such as cellulase or pectinase, sonication, grinding or the like. Then, insoluble matters are removed therefrom by filtration, centrifugation, etc. to thereby obtain a crude protein solution or a solution containing the primary and/or the secondary metabolite of the plant.

In order to further purify the protein of the invention from the above crude protein solution, conventional protein purification methods may be used. For example, ammonium sulfate salting out, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography, affinity chromatography or electrophoresis may be used independently or in an appropriate combination.

In order to recover the protein of the invention from plant organs or plant bodies, first, an extract of the useful substance is prepared by disrupting the plant organs or bodies by sonication or grinding. Subsequently, the above-described purification procedures may be followed.

4. Transit Peptides

(1) Specification of Transit Peptide Sequences

The transit peptide of the invention is a peptide comprising the amino acid sequence shown in SEQ ID NO: 17. The location of this amino acid sequence can be specified by sequence analysis of the cloned choline monooxygenase (CMO) gene. This amino acid sequence is encoded by the nucleotide sequence shown in SEQ ID NO: 16.

Once the amino acid sequence has been known, the transit peptide of the invention may be produced by chemical synthesis as described below.

(2) Chemical Synthesis of Transit Peptides

The transit peptide of the invention may be produced by conventional peptide synthesis techniques based on the amino acid sequence specified as described above. Either the liquid synthesis method or the solid synthesis method may be used. Such peptide synthesis may be

performed by any of the known methods (see, for example, Bodanszky, M. and M.A. Ondetti, Peptide Synthesis, Interscience Publishers, New York (1966); Schroeder and Luebke, The Peptide, Academic Press, New York (1965); F.M. Finn and K. Hofmann, The Proteins, Vol. 2, H. Nenrath and R. L. Hill (eds.), Academic Press Inc., New York (1976); N. Izumiya et al., Basics and Experiments in Peptide Synthesis, Maruzen Co., Tokyo (1985); H. Yajima, S. Sakakibara et al., Course of Biochemistry Experiment Lectures No. 1, The Japanese Biochemical Society (ed.), Tokyo Kagaku Dojin Co., Tokyo 1977; T. Kimura, 2nd Series: Course of Biochemistry Experiment Lectures No. 2, The Japanese Biochemical Society (ed.), Tokyo Kagaku Dojin Co., Tokyo 1987). Thus, the transit peptide of the invention can be obtained by, for example, the azide method, the acid chloride method, the acid anhydride method, the mixed acid anhydride method, the DCC method, the active ester method, the method using Woodward's reagent K, the carbonylimidazole method, the oxidation-reduction method, the DCC/HONB method, or the method using BOP reagent. Usually, the transit peptide may be synthesized with a commercial, automated peptide synthesizer.

The transit peptide of the invention can be prepared by ligating a peptide of interest to a peptide fragment of the transit peptide by condensation and then removing the protecting groups of the C-terminal α-carboxyl and N-terminal α-amino groups of the resultant product at the same time or in a stepwise manner.

After completion of the reaction, the thus prepared peptide can be recovered by a combination of peptide separation/purification techniques such as solvent extraction, distillation, partition, reprecipitation, recrystallization, column chromatography, high performance liquid chromatography, gel filtration, ion exchange chromatography and ion exchange chromatography.

The transit peptide of the invention may be obtained in the form of a metal salt; a salt made of the peptide and a base or basic compound; an inorganic acid addition salt; an organic salt; or the like. In particular, the transit peptide of the invention can be obtained as a pharmaceutically acceptable acid addition salt (e.g. a salt made of the peptide and an inorganic or organic acid). Specific examples of acid addition salts include salts made of the peptide and

inorganic acids such as hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid; or salts made of the peptide and organic acids such as acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid. Specific examples of basic salts include salts made of the peptide and inorganic bases such as sodium hydroxide, potassium hydroxide, ammonium hydroxide, magnesium hydroxide; or salts made of the peptide and organic bases such as caffeine, piperidine, trimethylamine, pyridine. Specific examples of metal salts include sodium salts, potassium salts, calcium salts and magnesium salts.

The salt of the transit peptide of the invention can be prepared using an appropriate acid such as hydrochloric acid or an appropriate base such as sodium hydroxide. For example, the peptide may be treated with such an acid or base in water or a liquid containing an inactive, water-miscible organic solvent such as methanol, ethanol or dioxane according to a standard protocol to thereby prepare a salt. The treatment temperature may be from 0 to 100℃; room temperature is preferable.

The biochemical and physicochemical properties of the transit peptide of the invention can be analyzed by mass spectrometric analysis, nuclear magnetic resonance, electrophoresis, high performance liquid chromatography, etc.

5. Construction of a Complex Composed of Gene of Interest and Transit Peptide Gene, and Induction of Substance Accumulation

In the present invention, a gene encoding the transit peptide (i.e. peptide having a function as a signal peptide) of the invention is ligated upstream of a gene of interest. A DNA complex can be obtained by digesting the gene of interest and the transit peptide gene with appropriate restriction enzymes and then ligating these two genes to each other using ligase. The thus ligated DNA is ligated to a vector predigested with appropriate restriction enzymes to thereby obtain a recombinant vector. The resultant vector is introduced into a host to thereby obtain a transformant. By culturing or cultivating the resultant transformant, an expression product of the gene of interest or a metabolite generated through the metabolism of the above expression

product by the host can be accumulated. Construction of the recombinant vector, selection of the host and transformation may be performed in the same manner as described above for the choline monooxygenase of the invention.

As the gene of interest, a gene encoding a polypeptide or enzyme may be given, but other genes may also be used. When the expression product of interest is an enzyme, the product of a reaction catalyzed by the enzyme or the intermediates and/or the final product (primary or secondary metabolite of plants) of a series of biosynthesis reactions following the above reaction may be accumulated as useful substances. Specific examples of such useful substances include a substance that confers environmental stress resistance, e.g. betaine. Further, when the gene of interest is a control gene (also called "master gene") in charge of the functional regulation of an entire reaction pathway (such as biosynthesis pathway) in mechanisms for transducing signals from phosphatases or G-protein, the intermediates and/or final product of the reaction pathway located downstream of the signal transduction of the above control gene may also be accumulated as useful substances. These substances may or may not be involved in environmental stress resistance.

When the transformant (in particular, transformed plant) thus obtained is cultured or cultivated under environmental stress conditions, an expression product of the gene of interest or a plant metabolite from the expression product is accumulated in the plant upon receipt of the environmental stress as a signal. Specific examples of environmental stresses include salt stress, dehydration stress, low temperature stress and high temperature stress.

Salt stress is loaded by adding sodium chloride to a specific hydroponic solution to give a concentration of 50-600 mM and culturing the transformed plant therein under usual conditions.

Dehydration stress is loaded by withdrawing the entire plant body from the soil or hydroponic solution and exposing it to the air, or by adding polyethylene glycol or the like to the hydroponic solution or medium.

High temperature or low temperature stress is loaded by rising or lowering the temperature of the incubator, green house, etc. in which the transformed plant is cultured or cultivated.

After loaded with stress, the plant is cultured or cultivated in the same manner as described earlier for the culturing of the transformant, to thereby obtain an environmental stress resistant plant. The term "environmental stress resistant" means the state of a plant that does not wither even under conditions which wither non-resistant plants or that is able to grow even under conditions which terminate the growth of non-resistant plants, when a particular stress (e.g. salt stress, dehydration stress) has been loaded.

In the present invention, it is possible to allow the expression of a choline monooxygenase-transit peptide complex by ligating a DNA encoding a transit peptide (SEQ ID NO: 16) to a DNA encoding choline monooxygenase (SEQ ID NO: 1, 3 or 5) as a gene of interest and incorporating the resultant construct into an expression vector. In this case, a choline monooxygenase gene with a DNA encoding a transit peptide is incorporated in the environmental stress resistant plant of the invention. When this plant is cultivated under an environmental stress as described above, accumulation of choline monooxygenase is induced. As a result, synthesis of betaine aldehyde from choline is catalyzed and, finally, betaine is accumulated in the plant. Such accumulation of betaine is significant in a sense that it can confer environmental stress resistance on the plant. For recovering betaine from plants, methods for purifying quaternary ammonium compounds may be employed.

PREFERRED EMBODIMENTS OF THE INVENTION

Hereinbelow, the present invention will be described more specifically with reference to the following Examples. However, the technical scope of the invention is not limited to these Examples. In the Examples, choline monooxygenase is expressed as "CMO" or "CMO protein" and choline monooxygenase gene as "cmo" or "cmo gene".

EXAMPLE 1

Preparation of RNA

Chenopodium album mature leaves (4 g) immediately after harvest were disrupted in liquid nitrogen with a blender. To the disrupted leaves, 20 ml of a guanidine thiocyanate solution (4.2 M guanidine thiocyanate, 25 mM sodium citrate dihydrate; immediately before use, 7 μ l of 2-mercaptoethanol and 5 mg of sodium lauroyl sarcosinate are added per milliliter of the solution) was added and shaken vigorously for 10 min at room temperature. The resultant mixture was centrifuged at 10,000 rpm for 10 min to obtain a supernatant, to which 1 g of CsCl was added per 2 ml. This supernatant (6-7 ml) was overlayered upon 4 ml of 5.7 M CsCl solution (5.7 M CsCl, 0.1 M EDTA (pH 7.5)) contained in an polyalomer tube and ultra-centrifuged at 35,000 rpm at 20 ℃ for 18 hr.

The resultant precipitate was dissolved completely in 5 ml of Tris-SDS solution (50 mM Tris-HCl (pH 9.0), 1% SDS). To this solution, 5 ml of phenol (pH 9.0) was added and shaken at room temperature for 10 min. Then, the resultant solution was centrifuged at 5,000 rpm at 20℃ for 10 min. To the resultant supernatant, 5 ml of phenol/chloroform was added and shaken at room temperature for 10 min. Then, the resultant solution was centrifuged at 5,000 rpm at 20 ℃ for 10 min. To the resultant supernatant, 5 ml of chloroform was added and shaken at room temperature for 10 min. Then, the resultant solution was centrifuged at 5,000 rpm at 20℃ for 10 min. To the resultant supernatant, 1/10 volume of 3 M NaOAc was added. Then, 2 volumes of EtOH was added thereto and mixed. The mixture was left at -20℃ for 30 min, followed by centrifugation at 10,000 rpm at 4℃ for 10 min. The resultant precipitate was dissolved in 1 ml of H_2O to thereby obtain RNA from *Chenopodium album* mature leaves.

EXAMPLE 2

Acquisition of a Gene Fragment by RT-PCR and Cloning of the Full-Length Gene

(1) Acquisition of a Gene Fragment by RT-PCR

A gene fragment was obtained using RT-PCR Kit (Stratagene) according to the protocol attached to the kit basically. PCR primers were designed based on the amino acid sequence of

spinach CMO.

Chenopodium album RNA (9.5 μg) was dissolved in 38 μl of DEPC-treated H_2O. Three microliters of random primer (100 ng/μl) was added thereto, followed by incubation at 65℃ for 5 min. Then, the solution was cooled slowly to room temperature. To this solution, 5 μl of 10x 1st strand buffer, 1 μl of RNase Block Ribonuclease Inhibitor (40 U/μl), 2 μl of 100 mM dNTPs and 1 μl of MMLV reverse transcriptase (50 U/μl) were added and reacted at 37 ℃ for 1 hr. After the reaction, the reaction solution was incubated at 90 ℃ for 5 min and then placed on ice. Using 5 μl of the thus obtained 1st strand cDNA solution as a template, the following PCR reaction solution was prepared and incubated at 91 ℃ for 5 min and at 54℃ for 5 min.

Composition of the PCR Reaction Solution:

1st strand cDNA solution	5 μl
10x Ex Taq buffer (Takara)	10 μl
dNTPs mix (2.5 mM each)	8 μl
Primer 1 (SEQ ID NO: 7)	100 pmol
Primer 2 (SEQ ID NO: 8)	100 pmol
Total volume	99.5 μl

Subsequently, 0.5 μl of Takara Ex Taq DNA polymerase (5 U/μl) was added to the reaction solution. Then, a PCR reaction was performed 30 cycles, one cycle consisting of denaturation at 91 ℃ for 1 min, annealing at 54 ℃ for 1 min and extension at 72℃ for 2 min. After the reaction, the reaction solution was subjected to agarose gel electrophoresis. A fragment of approx. 600 bp which was believed to be the product of interest was cut out from the gel, purified, cloned into pT7Blue T-Vector (Novagen) and sequenced to thereby obtain a *Chenopodium album* cmo gene fragment.

(2) Purification of mRNA

Purification of mRNA was performed using mRNA Purification Kit (Pharmacia) and

according to the protocol attached to the kit. Briefly, 0.9 mg of total RNA from *Chenopodium album* was dissolved in 1 ml of an elution buffer. The solution was heated at 65 ℃ for 5 min and then immediately ice-cooled. To this solution, 0.2 ml of a sample buffer was added, and the resultant solution was applied to an oligo (dT)-cellulose spin column pre-equilibrated with a high-salt buffer. After elution, the column was centrifuged at 350xg for 2 min. Subsequently, the column was washed by adding thereto 0.25 ml of a high-salt buffer and centrifuging at 350xg for 2 min; these washing operations were performed twice. Then, similar washing operations were performed 3 times using 0.25 ml of a low-salt buffer.

RNA was recovered by repeating the following operations 4 times: addition of 0.25 ml of an elution buffer preheated to 65℃ and centrifugation at 350xg for 2 min. The resultant RNA solution (1 ml) was column-purified again in the same manner as described above. To 1 ml of the resultant RNA solution, 100 μl of a sample buffer, 10 μl of a glycogen solution and 2.5 ml of EtOH were added, and the resultant solution was left at -20℃ for 2 hr. Then, the solution was centrifuged at 14,000 rpm at 4 ℃ for 10 min. The precipitate was dissolved in 20 μl of H_2O, followed by the determination of absorbance.

Thus, 21 μg of mRNA was obtained.

(3) Synthesis of cDNA

(3-1) Synthesis of 1st Strand cDNA

Water was added to 1 μg of *Chenopodium album* mRNA and 1 μl of cDNA synthesis primer (10 μM) to give a 5 μl solution, which was heated at 70℃ for 2 min and immediately cooled on ice for 2 min. To this solution, 2 μl of 5x 1st strand buffer, 1 μl of dNTPs mix (10 mM), 1 μl of MMLV reverse transcriptase (100 U/μl) and 1 μl of H_2O were added. After heating at 42℃ for 1 hr, the solution was immediately cooled on ice.

(3-2) Synthesis of 2nd Strand cDNA

Ten microliters of the 1st strand reaction solution, 16 μl of 5x 2nd strand buffer, 1.6 μl of dNTPs mix (10 mM), 4 μl of 20x 2nd strand enzyme cocktail and 48.4 μl of H_2O were mixed gently on ice, and the mixture was incubated at 16℃ for 45 min. Then, the reaction

was terminated by adding thereto 4 μl of a mixture of EDTA and glycogen. To the reaction solution, 100 μl of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) was added and vortexed. Then, the solution was centrifuged at 14,000 rpm for 10 min. To the resultant supernatant, 100 μl of a mixture of phenol:isoamyl alcohol (24:1) was added and vortexed, followed by centrifugation in the same manner as described above. To the resultant supernatant, 1/2 volume of 4 M ammonium acetate and 2.5 volumes of EtOH were added, followed by centrifugation at 14,000 rpm for 20 min. The precipitate was rinsed with 80% EtOH, vacuum-dried and then dissolved in 10 μl of H_2O. Two microliters of this solution was subjected to 0.8% agarose gel electrophoresis for confirmation. Thus, double-stranded (ds) cDNA was obtained.

To 5 μl of the ds cDNA, 2 μl of Marathon cDNA adaptor (10 μM), 2 μl of 5x DNA ligation buffer and 1 μl of T4 DNA ligase (1 U/μl) were added and incubated at 16 ℃ overnight. After deactivation of the ligase by heating at 70℃ for 5 min, 1 μl of the reaction solution was diluted with 250 μl of Tricine-EDTA buffer. The diluted solution was heated at 94℃ for 2 min and then cooled on ice for 2 min, to thereby obtain an adaptor-ligated cDNA for use in RACE PCR.

(4) 5' and 3' RACE-PCR

On 5 μl of the adaptor-ligated cDNA as a template, a PCR was performed using Advantage Klen Taq polymerase (Clontech). Then, 5 μl of the reaction solution was subjected to 0.8% agarose gel electrophoresis for confirmation of the amplified product.

For 5' RACE-PCR, primer 3 (SEQ ID NO: 9) was used. For 3' RACE-PCR, primer 4 (SEQ ID NO: 10) was used.

Composition of the PCR Reaction Solution:

H_2O	36 μl
10 mM dNTPs mix	1 μl
50x Klen Taq polymerase mix	1 μl
10x Klen Taq buffer (Clontech)	5 μl

Adaptor-ligated cDNA	5 μl
10 μM AP1 primer (Clontech)	1 μl
10 μM Primer (primer 3 or primer 4)	
	Total: 50 μl

PCR conditions were as follows: first denaturation at 94 ℃ for 1 min, then 5 cycles of reaction at 94 ℃ for 30 sec and at 72 ℃ for 4 min; then 5 cycle of reaction at 94℃ for 30 sec and at 70 ℃ for 4 min; and finally 25 cycles of reaction at 94 ℃ for 30 sec and at 68 ℃ for 4 min.

An approx. 1.3 kbp band which was believed to be the 5' RACE product and an approx. 1.2 kbp band which was believed to be the 3' RACE product were confirmed. Each of these bands was cut out from the agarose gel, purified and cloned into pT7Blue T-vector. The nucleotide sequence of each clone was determined by the dye-terminator method and analyzed. As a result, it was found that three cmo genes of type A (SEQ ID NO: 1), type B (SEQ ID NO: 3) and type C (SEQ ID NO: 5) exist.

The amino acid sequences encoded by type A, type B and type C genes are shown in SEQ ID NOS: 2, 4 and 6, respectively.

(5) Acquisition of the Full-Length Type C cmo Gene

Among the three cmo genes, the type C gene was selected for future analysis. Then, the inventors isolated the full-length nucleotide sequence of type C cmo gene.

Briefly, a PCR reaction was performed using a SmaI site-added primer (primer 5)(SEQ ID NO: 11), an XbaI site-added primer (primer 6)(SEQ ID NO: 12) and KOD DNA polymerase (Toyobo), and the amplified product was ligated to pT7Blue T-vector (Novagen). The PCR was performed 30 cycles, one cycle consisting of denaturation at 94 ℃ for 1 min, annealing at 60 ℃ for 1 min and extension at 72℃ for 2 min.

Composition of the PCR Reaction Solution:

Primer concentration 20 pmol	
2 mM dNTPs	5 μl
Adaptor-ligated cDNA	10 μl
25 mM $MgCl_2$	2 μl
KOD DNA polymerase (2.5 U/μl)(Toyobo)	1 μl
10x PCR buffer (Toyobo)	5 μl
	Total: 50 μl

The nucleotide sequence of the amplified product was determined by the dye-terminator method to thereby confirm that the product was type C gene. This was designated "pT7cmo".

(6) Preparation of Antibodies to CMO Protein

In order to analyze the expression of CMO protein, antibodies to CMO protein were prepared using Xpress System (Invitrogen).

Briefly, 5' primer (primer 7)(SEQ ID NO: 13) to which BamHI site was added and 3' primer (primer 8)(SEQ ID NO: 14) to which a KpnI site was added were prepared for amplifying the code region of the protein excluding the transit peptide. Using these primers, a PCR was performed to thereby amplify an approx. 1.2 kbp fragment. The PCR was performed 30 cycles, one cycle consisting of denaturation at 94℃ for 1 min, annealing at 60 ℃ for 1 min and extension at 72℃ for 2 min.

Composition of the PCR Reaction Solution:

H_2O	78 μl
4 mM dNTPs mix	8 μl
Ex Taq (5 U/μl) (Takara)	0.5 μl
10x Ex Taq buffer (Takara)	10 μl
pT7cmo (1 ng/μl)	1 μl
10 μM Primer (primer 7 + primer 8)	
	Total: 100 μl

The amplified product was ligated to pT7Blue T-vector (Novagen) and designated "pT7cmoA". pT7cmoA and pTrcHis were digested with restriction enzymes BamHI and KpnI, and separately subjected to 0.8% agarose gel electrophoresis. Using Gene Clean Spin Kit (BIO 101), a cmo gene fragment of approx. 1.2 kbp and a vector fragment of approx. 4.4 kb were recovered from the gel according to the manual attached to the kit. After purification, these two fragments were ligated to each other in a reaction system of 50 μl, using DNA Ligation Kit (Takara) utilizing T4 DNA ligase, and the thus ligated DNA is referred to as pTHC. The ligated DNA was introduced into *E. coli* (JM 109; Takara) to thereby prepare a fusion protein expression vector pTHC.

The thus prepared pTHC was purified by the miniprep method and introduced into *E. coli* (TOP10; Invitrogen) according to the manual attached to TOP10. The pTHC-introduced *E. coli* was cultured according to the manual attached to Xpress System (Invitrogen). From 400 ml of the resultant culture liquid, histidine-labelled CMO protein was prepared and applied to a Probond resin column (Invitrogen). Thus, approx. 10 mg of the protein was immobilized in the column. From this column, 350 mM imidazole fraction was recovered, followed by removal of imidazole with PB-10 (Pharmacia). The resultant solution was applied to a Probond resin column (Invitrogen) again to immobilize the protein. Two hundred units of enterokinase was mixed with approx. 4.5 mg of the immobilized fusion protein and shaken at room temperature for 10 hr. In 4 ml of the eluate from this column, the presence of a 43 kDa protein (equivalent to 1.6 mg of CMO mature protein) was confirmed by SDS-PAGE.

After purification, this CMO protein was administered to rabbits as antigen to prepare anti-sera, which were used as antibodies.

EXAMPLE 3

Construction of Expression Vectors

For the purpose of expressing *Chenopodium album* cmo gene in tobacco, two types of

expression vectors were prepared. One was pBIcmo comprising the DNA shown in SEQ ID NO: 16 encoding the transit peptide (SEQ ID NO: 17), and the other was pBIcmoS not comprising the DNA encoding the transit peptide.

First, a SmaI site-added primer (primer 9) (SEQ ID NO: 15) was prepared in order to amplify a cmo gene sequence without the region encoding the transit peptide.

Using this primer as 5' primer and primer 6 (SEQ ID NO: 12) as 3' primer, a PCR reaction was performed to amplify a gene fragment without the region encoding the transit peptide. The amplified fragment was ligated to pT7Blue T-vector (Novagen), which was designated pT7cmoS. The PCR was performed 30 cycles, one cycle consisting of denaturation at 94 ℃ for 1 min, annealing at 60 ℃ for 1 min and extension at 72℃ for 2 min.

Composition of the PCR Reaction Solution:

H_2O	78 μl
4 mM dNTPs mix	8 μl
Ex Taq (5 U/μl) (Takara)	0.5 μl
10x Ex Taq buffer (Takara)	10 μl
pT7cmo (1 ng/μl)	1 μl
10 μM Primer (primer 9 + primer 6)	
	Total: 100 μl

pT7cmo and pT7cmoS were separately digested with restriction enzymes SacI and SmaI, and subjected to agarose gel electrophoresis. Then, an approx. 1.2 kbp band and an approx. 1.4 kbp band were cut out from the gel and purified. pBI121 (purchased from Clontech) was digested with restriction enzymes SacI and SmaI, and then an approx. 11 kbp band was cut out and purified in the same manner. This band was ligated to each of the above-mentioned fragments to thereby prepare pBIcmo and pBIcmoS.

EXAMPLE 4

Gene Transfer into Tobacco

(1) Transformation of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens LBA4404 (purchased from Clontech) was cultured in L medium containing 250 μ g/ml streptomycin an 50 μ g/ml rifampicin at 28 ℃. A cell suspension was prepared from the culture according to the method of Nagel et al. (Microbiol. Lett., 67:325, 1990). Then, pBIcmo and pBIcmoS were separately introduced into the above-mentioned strain.

(2) Transfer of the Polynucleotide Encoding CMO into Tobacco Cells

Using the transformed *Agrobacterium tumefaciens* obtained in (1) above, transformation of *Nicotiana tabacum* cv. SR1 was performed according to the method of Horschet, et al. (Science, 277: 1229-1231, 1985).

In each of the following Examples, those lines which were homozygous with respect to the transgene were selected from R1 generation of the cmo gene-transferred tobacco that had been obtained through redifferentiation, and used in experiments. In the cmo gene-transferred tobacco as obtained above, CMO protein is constantly expressed since the polynucleotide of the invention encoding CMO is under the control of CaMV35S promoter which is a high expression promoter.

EXAMPLE 5

Method of Tobacco Cultivation

Tobacco seeds were sterilized by shaking them in 40 ml of 10% aqueous solution of sodium hypochlorite (Nacalai Tesque) supplemented with 10 μ l of Triton X-100 for 10 min and then washed with 500 ml of sterilized water in parts. The sterilized tobacco seeds were grown in 1/2 MS medium (sucrose concentration: 1.5%), which is a medium containing one half (1/2) of each of the components of Murashige and Skoog (MS) medium (Murashige et al., Physiol. Plant. 15:473-497, 1962), at 25℃ under conditions of 16 hr light/8hr dark for 2 weeks.

Thereafter, the resultant seedlings were transplanted to square-shaped pots containing 1/2 MS medium and subjected to various experiments.

(1) Western Blot Analysis

The expression of CMO protein was examined at the protein level using the above-described cmo gene-transferred tobacco and a wild-type, non-recombinant tobacco SR1.

Briefly, completely unfolded upper leaves (0.2 g) were taken from cmo gene-transferred tobacco (pBIcmo 4-2 line and pBIcmoS 53-1 line) and non-recombinant *Nicotiana tabacum* cv. SR1. The sample was disrupted in liquid nitrogen, suspended in 0.4 ml of an extraction buffer (1% SDS, 0.1 M $NaHCO_3$, 5% 2-mercaptoethanol) and boiled for 5 min. Then, the sample was centrifuged at 15,000 rpm for 5 min at room temperature to obtain the supernatant as a protein extract. This protein extract was separated by SDS-PAGE and transferred onto a nylon membrane (Imobilon; Millipore). This membrane was incubated with the above-described antibody to CMO (5000-fold dilution of the anti-serum) and washed. The membrane was further incubated with a secondary antibody of 3000-fold dilution [affinity-purified goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate; BioRad] and washed, followed by detection of CMO with a coloring solution (Konica Immunostain HRP-1000; Konica).

The results of the Western blotting are shown in Fig. 1. The existence of an immune responsive protein of 43 kDa corresponding to CMO was confirmed. In pBIcmo-transferred tobacco, it was shown that accumulation of CMO protein from which the transit peptide had been removed was increased by several times when 150 mM NaCl stress was loaded for 1 day. In pBIcmoS-transferred tobacco, no induction of protein accumulation was observed when 150 mM NaCl stress was loaded. From these results, it was indicated that a polypeptide sequence corresponding to the *Chenopodium album* CMO transit peptide promotes protein accumulation in response to salt stress.

(2) Determination of Betaine Accumulation in Transformed Plants

Betaine contents in plant leaves were calculated by measuring NMR spectra of quaternary

ammonium compounds (Wall, J. et al., Analyt. Chem. 32:870-874, 1960). One gram each of leaves from the wild-type plant and the transformed plant was powdered in liquid nitrogen using a ceramic motor. The resultant powder was suspended in 4 ml of 1.0 M H_2SO_4, which was then shaken at 25 ℃ for 24 hr. After removal of insoluble matters, the suspension was centrifuged at 1000xg for 10 min to recover a supernatant. To 1 ml of this supernatant, 0.4 ml of KI-I_2 solution was added and shaken at 4 ℃ for 80 min. The resultant solution was centrifuged at 13,000xg to thereby recover periodite-addition products of betaine, choline or the like. These products were dissolved in 0.6 ml of D20 (EURISO-TOP) containing t-butyl alcohol (Nacalai Tesque) as an internal standard, followed by measurement of 1H-NMR spectra.

As a result, two major peaks of betaine and choline were observed. The integrated value for the betaine peak was used for the quantitative determination of betaine concentration.

Tobacco plants grown for 2 weeks after sowing were transplanted to square-shaped pots containing 1/2 MS medium supplemented with 20 mM choline (Nacalai Tesque) and 100 mM NaCl. After 2-week cultivation, samples were collected and used for the quantitative determination of betaine.

As a result, while only choline was observed in the wild-type plants, both betaine and choline were observed in the transformed plants. As shown in Fig. 2, the betaine content of pBIcmo4-2 plant was 2.0 μ g/g fresh weight. These results indicated that transgenic plants expressing the CMO mature protein have ability to accumulate betaine.

All publications, patents and patent applications cited herein are incorporated by reference in their entirety.

According to the present invention, choline monooxygenase and the gene thereof are provided. The gene is applicable to the breeding of those plants which are highly tolerant to dry or saline soil. Also, the creation of such plants using the above gene will be helpful to restore plant cultivation in wasteland resulted from dry or saline soil, or to increase the yields of crops in areas of dry soil or saline soil.